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Lack of increased genetic damage in 1,3-butadiene-exposed Chinese workers studied in relation to *EPHX1* and *GST* genotypes

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Abstract

1,3-Butadiene (BD) is an important industrial chemical and pollutant. Its ability to induce genetic damage and cause hematological malignancies in humans is controversial. We have examined chromosome damage by fluorescence in situ hybridization (FISH) and mutations in the *HPRT* gene in the blood of Chinese workers exposed to BD. Peripheral blood samples were collected and cultured from 39 workers exposed to BD (median level 2 ppm, 6 h time-weighted average) and 38 matched controls in Yanshan, China. No difference in the level of aneuploidy or structural changes in chromosomes 1, 7, 8, and 12 was detected in metaphase cells from exposed subjects in comparison with matched controls, nor was there an increase in the frequency of *HPRT* mutations in the BD-exposed workers. Because genetic polymorphisms in glutathione S-transferase (*GST*) enzymes and microsomal epoxide hydrolase (EPHX1) may affect the genotoxic effects of BD and its metabolites, we also related chromosome alterations and gene mutations to *GSTT1*, *GSTM1* and *EPHX1* genotypes. Overall, there was no effect of variants in these genotypes on numerical or structural changes in chromosomes 1, 7, 8 and 12 or on *HPRT* mutant frequency in relation to BD exposure, but the *GST* genotypes did influence background levels of both hyperdiploidy and *HPRT* mutant frequency. In conclusion, our data show no increase in chromosomal aberrations or *HPRT* mutations among workers exposed to BD, even in potentially susceptible genetic subgroups. The study is, however, quite small and the levels of BD exposure are not extremely high, but our findings in China do support those from a similar study conducted in the

Abbreviations: BD, 1,3-butadiene; EPHX1, microsomal epoxide hydrolase; FISH, fluorescence in situ hybridization; GST, glutathione S-transferase; *HPRT*, hypoxanthine-guanine phosphoribosyl transferase gene; SCEs, sister chromatid exchanges; S.D., standard deviation; S.E., standard error; TWA, time-weighted average

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Czech Republic. Together, these studies suggest that low levels of occupational BD exposure do not pose a significant risk of genetic damage.

Keywords: 1,3-Butadiene; Chromosomal aberrations; Fluorescence in situ hybridization; *HPRT*; GSTs; EPHX1; Genotypes

1. Introduction

1,3-Butadiene (BD) is widely used in the production of rubber and thermoplastic resins [1]. It is also a common environmental pollutant being found in automobile exhaust, cigarette smoke, and in community air [2]. BD is now classified as a probable human carcinogen, but several regulatory agencies have recently considered raising its status to an established human carcinogen. However, the evidence of carcinogenicity in humans is inconsistent. The strongest evidence comes from a study of workers in the US and Canada styrene–butadiene rubber industry, which showed excesses of leukemia with risk increasing in direct relation to BD exposure dose [3,4]. In contrast, in monomer production workers exposed to a relatively low level of pure BD (<2 parts per million, ppm), no significant associations between BD exposures and leukemia risk have been observed [5,6]. Overall, the epidemiological findings in BD-exposed workers suggest but do not prove carcinogenicity for humans.

To provide mechanistic information on the potential carcinogenicity of BD in humans, we [7–9] and others [10–15] have investigated genotoxic effects in BD-exposed workers. In a study at a polybutadiene production facility in China, we examined a broad spectrum of genotoxic and other potential effects of BD. Here, we report in detail on part of that investigation: the analysis of cytogenetic changes in chromosomes 1, 7, 8 and 12 using fluorescence in situ hybridization (FISH) and *HPRT* (hypoxanthine-guanine phosphoribosyl transferase) mutations and their possible correlation with polymorphisms in genes encoding the glutathione S-transferases (*GSTs*) and microsomal epoxide hydrolase (*EPHX1*), which metabolize potentially genotoxic BD epoxides. We have previously reported data on *HPRT* mutant frequency [7], but not in relation to these genotypes.

The genotype of *EPHX1* was examined because the recent report by Abdel-Rahman et al. showed that the Y113H polymorphism in this gene was correlated with increased *HPRT* gene mutation frequency and BD exposure [16]. An increased frequency of *HPRT* mutations (M_f) was observed in the lymphocytes of workers exposed to BD (>150 ppb) who also had at least one His allele (H/H or Y/H genotypes). The M_f among these highly exposed workers was three-fold higher compared with individuals with the Y/Y genotype ($P < 0.001$). In the present study, we have also examined another functional polymorphic variant in *EPHX1* at position 139 (H139R), in order to provide a more complete phenotypic picture of this important enzyme in BD metabolism. In addition, we have examined common polymorphisms in *GST* Mu (*GSTM1*) and Theta (*GSTT1*), which are also potentially involved in the detoxification of BD epoxides.

We chose to study damage to chromosomes 1, 7, 8 and 12 because chromosome 1 is the largest chromosome with theoretically the largest amount of DNA available for adduct formation and mutation; chromosomes 7 and 8 because these are commonly altered in acute myeloid leukemia and related disorders; chromosome 12 because we have previously shown that this chromosome is highly sensitive to damage by mono- and di-epoxide metabolites of BD in human lymphocytes [17]. We also utilized a T-lymphocyte cloning assay to examine *HPRT* gene mutations. We hypothesized that having low EPHX1 activity and no GSTM1 or T1 activity due to common polymorphisms in these genes would enhance genetic damage produced by BD exposure. In fact, we found that the genotypes of *EPHX1* and *GSTs* had no effect on the level of genetic damage produced by BD.

2. Materials and methods

2.1. Study population

Identification of facility and enrollment of study subjects has been previously described in detail [7]. At

a polybutadiene rubber production facility in Yanshan, China, the purification of BD from an initial hydrocarbon stream occurred at two sites: the DMF facility, where initial distillation and extraction occurred using a proprietary dimethyl formamide (DMF) process; and the recovery facility, where final distillation occurred. Three groups of workers with potentially high exposure were identified. DMF process analysts sampled process lines and analyzed product by gas chromatography at the DMF unit, while polymer process analysts carried out these tasks at the recovery and polymerization units. The third group of exposed workers were process operators at the recovery facility who carried out routine minor maintenance and, as needed, major repair operations. After the purposes of the study and procedures were explained and informed consent was obtained, 39 exposed workers were included for study. For comparison, 38 unexposed subjects from the same factory were enrolled. Because the production process was enclosed, general environmental emissions were limited. The unexposed subjects were age (5-year intervals) and gender matched in groups to the exposed. A group of Chinese and US scientists administered a brief questionnaire to the study subjects regarding work history, selected medical conditions, and tobacco use.

2.2. Exposure assessment

During the 6 h work shift, personal samplers were used for collecting air at the breathing zone by drawing atmospheres through charcoal tubes using individual pumps. Two traps were combined in series to ensure that all BD was retained. During the study, numerous grab samples at the breathing zone were also taken, using 50 ml glass collection syringes. In addition, canister samples were collected at five locations.

2.3. Blood cell cultures

Whole blood collected in a vacutainer with the anticoagulant heparin was cultured in RPMI-1640 medium supplemented with 15% fetal bovine serum, 1% penicillin–streptomycin, 1% L-glutamine (Gibco, Grand Island, NY) and 1% phytohemagglutinin-P (Pharmacia, Piscataway, NJ). Blood cells were incubated at 37 °C in a 5% CO_2 moist atmosphere and harvested at 72 h after culture initiation. Colcemid (0.1 μg/ml) was added 4 h prior to harvest to obtain a sufficient number of metaphase spreads (for metaphase FISH only). After hypotonic treatment (0.075 M KCl) for 30 min at 37 °C, the cells were fixed three times with freshly made Carnoy's solution (methanol:glacial acetic acid = 3:1). The fixed cells were then dropped onto pre-labeled glass slides, allowed to air dry, and stored at −20 °C under a nitrogen atmosphere. Prepared slides were later shipped on dry ice to the United States.

2.4. Fluorescence in situ hybridization

A total of four chromosomes were examined using two different types of probes purchased from Oncor, Inc. (Gaithersburg, MD) and Vysis, Inc. (Downers Grove, IL). The centromeres of chromosomes 1 and 7 were targeted by α-satellite DNA probes and chromosomes 8 and 12 were painted along their whole lengths by painting probes. The signals on chromosomes 1 and 8 were detected as green and those on 7 and 12 as red. A simplified denaturation and hybridization procedure was performed automatically by the HyBrite Denaturation/Hybridization system from Vysis, Inc. (Downers Grove, IL). The centromere and painting probes were mixed well, then applied onto slides and coverslipped. The denaturation temperature was set at 72 °C and time at 7 min. Slides remained in the moist environment of the system at 37 °C for 45–68 h in order to obtain optimal signals. Slides were then postwashed in 1× SSC at 70 °C for 5 min and in phosphate buffer three times at room temperature.

Hybridization signals were detected in a dual-color detection solution with 10 μg/ml anti-digoxigenin (Boehringer–Mannheim, Indianapolis, Indiana) and 10 μg/ml FITC-avidin (Vector, Burlingame, CA) in phosphate buffer for 30 min at 37 °C. After the slides were washed three times for 2 min at a time in phosphate buffer with intermittent agitation at room temperature, the nuclei of cells were counterstained with a blue fluorescent dye 4,6-diamino-2-phenylindole (DAPI, 0.1 μg/ml) (Sigma, St. Louis, MO) prepared in a mounting medium (Vector, Burlingame, CA). The hybridization signals were viewed using a fluorescence microscope equipped with epifluorescent

illumination and a 100× oil immersion lens. A triple-bandpass filter for DAPI/FITC/Texas Red (excitation at 405, 490 and 570 nm; emission at 460, 525 and 635 nm) was used. All the stained slides were randomized and coded prior to scoring. For efficiency, all scorable metaphase spreads on each slide were analyzed and a minimum of 100 cells per subject was scored. Metaphase cells were considered scorable if they met specific criteria [18,19]. The total number of scored metaphase spreads from the 77 subjects was 17,069 (average 222 cells per subject).

2.5. HPRT mutation assay

The *HPRT* mutant frequency (M_f) was determined by the T cell cloning assay with cryopreserved cell samples as described previously [7]. Briefly, cryopreserved cells were thawed and incubated in medium containing 1 μg/ml PHA (HA17, Wellcome Diagnostics) for 36–40 h to achieve mitogen stimulation. Washed cells were then plated in growth medium (RPMI-1640 containing 20% nutrient medium HL-1, 5% defined supplemented bovine calf serum, 10–20% LAK supernatant containing 0.125 μg/ml PHA) and 1×10^4 irradiated human lymphoblastoid feeder cells per well. After an incubation of 10–16 days, growing colonies were determined by use of an inverted phase contrast microscope. The cloning efficiencies (CE) are calculated by Poisson relationship: $CE = -\ln P_o/x$, where P_o is the fraction of wells negative for colony growth and x is the average number of cells originally inoculated per well by limiting dilution. The thioguanine-selected CE divided by the mean unselected CE yields the M_f. The results have been reported in detail previously [7].

2.6. Genotyping of GSTs and EPHX1

GSTT1 and GSTM1: Target DNA (50–100 ng) was obtained from heparinized whole blood. PCR reactions were carried out in 50 μl volume containing 10 mM Tris–HCl, pH 8.3, 50 mM KCl, 1 mM 2-mercaptoethanol, 1% glycerol, 1 mM $MgCl_2$, 0.2 mM dNTPs and 2.5 Unit Amplitaq. The primers for *GSTT1* were 5′-TTCCTTACTGGTCCTCACATCTC-3′ and 5′-TCACCGGATCAT-GGCCAGCA-3′, and for *GSTM1* the primers were 5′-GTGCCCTACTTGA-TTGATGGG-3′ and 5′-CTGGATTGTAGCAGAT-CATGC-3′. PCR products were electrophoresed on 2% agarose gels and the diagnostic bands were visualized using ethidium bromide staining. Control amplifications were run in all lanes using universal primers for actin.

EPHX1: DNA was isolated from EDTA buffy coats using a QIAamp DNA blood mini kit (Qiagen Inc., Valencia, CA). PCR primers as described by Smith and Harrison [20], were used to detect the two *EPHX1* mutations, in separate PCR assays. PCR reactions were performed with 50 ng of genomic DNA in a 25 μl reaction mixture containing 1X Amplitaq Gold buffer II (Perkin Elmer, Foster City, CA), 1.5 mM $MgCl_2$, 200 mM of each dNTP, 12.5 pmol of each primer, and 0.625 U Amplitaq Gold enzyme. The PCR conditions were 94 °C for 10 min, followed by 40 cycles of 94 °C, 1 min, 56 °C, 1 min and 72 °C for 1 min, and a final extension step of 72 °C for 10 min.

Each PCR product (15 μl) was digested to completion with *EcoRV* (exon 3) or *RsaI* (exon 4) following supplier's directions (Promega), resolved on a 3% agarose gel, stained with ethidium bromide and visualized with UV light. Wild-type for exon 3 yields 140 and 22 bp fragments while the uncut variant allele is 162 bp. For exon 4 the wild-type does not cut with *RsaI* and remains a 210 bp fragment, while the mutant allele generates two fragments of 164 and 46 bp.

Specific primers for direct sequence analysis of the *EPHX1* exon 3 mutation were as described [21]. PCR was performed as above and products were purified using QIAquick PCR purification kit (Qiagen Inc., Valencia, CA) prior to sequencing.

2.7. Statistical analyses

Nonparametric procedures were used for statistical analysis, including the Spearman correlation test, the Wilcoxon Rank Sum *W* test for independent samples, and the χ^2-test. For multivariate analyses of studied markers, linear regression analyses were carried after transformation to the natural log (ln). Analyses were carried out using the SPSS statistical package [22]. Two-sided *P*-values were calculated with *P*-values of <0.05 considered adequate to reject the null hypothesis.

3. Results

3.1. Demographic characteristics of the study subjects

Exposed workers ($n = 39$) and controls ($n = 38$) had similar demographic characteristics (Table 1). None of the women in either group smoked, but more than 75% of men in both the exposed and control groups were smokers. The levels of BD exposure were measured in three different ways: (a) in air as 6 h time-weighted average (TWA); (b) in urine as a metabolite of BD, mercapturic acid butanediol (M1); (c) in blood as a hemoglobin *N*-(2,3,4-trihydroxybutyl)valine (THBVal) adduct. All three measurements have previously been shown to correlate with one another [8] and were consistently increased in BD-exposed workers (Table 1). Exposed males had worked in their respective facilities for a mean of 8.2 ± 6.3 years; females had worked for 6.7 ± 5.7 years.

Table 1
Selected demographic characteristics of study subjects

	Control ($n = 38$)	Exposed ($n = 39$)
Sex		
Male (%)	14 (37%)	15 (38%)
Female	24	24
Age		
Male	31.6 ± 4.8[a]	28.5 ± 6.0
Female	30.8 ± 6.1	27.5 ± 6.1
Tobacco use[b]		
n (%)	11 (79)	13 (87)
Pack-year	7.0 ± 5.2	6.1 ± 8.4
Exposure level		
BD in air[c]		
Median	0 ($n = 14$)	2.0[d] ($n = 39$)
Mean	0	44.0[d]
Ml in urine[e]		
Median	0.55 ($n = 4$)	1.3 ($n = 17$)
Mean	0.49	6.2
THBVal adducts[f]		
Median	37.6 ($n = 25$)	74.1[d] ($n = 33$)
Mean	39.3	88.3[d]
Exposure duration (year)		
Male	0	8.2 ± 6.3
Female	0	6.7 ± 5.7

[a] Mean ± S.D.
[b] In males only; no female smokers.
[c] Median and mean level (ppm) of 1,3-butadiene in air as 6 h TWA.
[d] $P < 0.0001$ by Wilcoxon test for independent samples.
[e] Median and mean level (μg/mg creatinine) mercapturic acid butanediol in urine.
[f] Median and mean level (pmol/g) of hemoglobin *N*-(2,3,4-trihydroxybutyl)valine adducts.

Table 2
Chromosomal damage among workers exposed to 1,3-butadiene

Numerical and structural chromosome changes	1,3-Butadiene exposure	
	Control ($n = 38$)	Exposed ($n = 39$)
Monosomy		
Chromosome 1	1.95 ± 0.29[a]	1.50 ± 0.30
Chromosome 7	2.66 ± 0.38	2.13 ± 0.36
Chromosome 8	2.79 ± 0.39	2.26 ± 0.35
Chromosome 12	2.94 ± 0.43	2.56 ± 0.37
Trisomy		
Chromosome 1	0.12 ± 0.04	0.09 ± 0.04
Chromosome 7	0.16 ± 0.06	0.28 ± 0.07
Chromosome 8	0.11 ± 0.04	0.17 ± 0.05
Chromosome 12	0.13 ± 0.05	0.10 ± 0.05
Tetrasomy		
Chromosome 1	0.63 ± 0.11	0.64 ± 0.16
Chromosome 7	0.60 ± 0.10	0.67 ± 0.17
Chromosome 8	0.61 ± 0.10	0.66 ± 0.17
Chromosome 12	0.60 ± 0.10	0.66 ± 0.17
Translocations		
t(8;?)[b]	0.07 ± 0.04	0.06 ± 0.03
t(12;?)[b]	0.09 ± 0.04	0.12 ± 0.06
t(8;12)	0.08 ± 0.05	0 ± 0
Total structural changes		
Chromosome 8	0.17 ± 0.07	0.08 ± 0.04
Chromosome 12	0.23 ± 0.07	0.18 ± 0.07

[a] Mean frequency (%) ± standard error of mean.
[b] Translocations between chromosome 8 or 12 and another unidentified chromosome.

3.2. Aneuploidy of chromosomes 1, 7, 8, and 12

Levels of monosomy of chromosomes 1, 7, 8, and 12 were not increased in exposed workers in comparison with controls (Table 2). In fact, monosomy levels were slightly higher among controls. There were no differences in levels of trisomy 1, 7, 8, and 12 between controls and exposed, although trisomy 7 was elevated in the exposed. Likewise, no difference was found in tetrasomies of all four chromosomes between

controls and exposed. Virtually the same level of tetrasomy was found among all four chromosomes in exposed, as well as in controls. Thus, the tetrasomy detected in chromosomes 1, 7, 8, and 12 was actually tetraploidy.

3.3. Structural changes in chromosomes 8 and 12

Structural changes, including translocations, deletions, and breakage on chromosomes 8 and 12, were readily detected since these two chromosomes were painted. The three major types of translocation detected were t(8;?), t(12;?), and t(8;12). No increased levels of these translocations were detected in exposed compared to controls (Table 2). Further, no difference was detected in total structural changes, which included all types on chromosomes 8 and 12, between exposed and controls. In fact, levels of total structural changes were slightly higher in controls than exposed and on chromosome 12 than on 8.

3.4. Effects of GSTT1 and GSTM1 genotypes

The genotypes of *GSTT1* and *GSTM1* were analyzable in most of our study subjects. Thirty-three of 38 controls and 38 of 39 exposed were typed for

Table 3
Effect of *GSTT1* and *GSTM1* genotype on chromosomal damage and *HPRT* mutation (mean frequency (%) ± standard error of mean)

	Control ($n = 33/38$)		Exposed ($n = 38/39$)		Total ($n = 71$)	
	Null[a] ($n = 13$)	Present[a] ($n = 20$)	Null ($n = 14$)	Present ($n = 24$)	Null ($n = 27$)	Present ($n = 44$)
(a) GSTT1 genotype						
Hyperdiploidy						
Chromosome 1	1.09 ± 0.28	0.55 ± 0.09	0.79 ± 0.40	0.73 ± 0.16	0.93 ± 0.24	0.65 ± 0.09
Chromosome 7	1.07 ± 0.27	0.61 ± 0.14[b]	1.0 ± 0.46	0.95 ± 0.20	1.03 ± 0.27	0.80 ± 0.13
Chromosome 8	0.96 ± 0.25	0.53 ± 0.10	0.93 ± 0.45	0.81 ± 0.16	0.94 ± 0.26	0.68 ± 0.10
Chromosome 12	1.01 ± 0.26	0.61 ± 0.12	0.73 ± 0.40	0.82 ± 0.18	0.86 ± 0.24	0.72 ± 0.11
Structural changes						
Chromosome 8	0.17 ± 0.15	0.19 ± 0.08	0 ± 0	0.09 ± 0.05	0.08 ± 0.07	0.14 ± 0.05
Chromosome 12	0.37 ± 0.16	0.16 ± 0.09	0.20 ± 0.10	0.15 ± 0.10	0.28 ± 0.09	0.15 ± 0.07[c]
HPRT ($M_f \times 10^{-6}$)[d]	17.73 ± 3.75	19.44 ± 3.98	26.79 ± 8.09	18.99 ± 1.85	23.17 ± 5.08	19.20 ± 2.08
n_{HPRT}[e]	8	17	12	19	20	36
	Control ($n = 33/38$)		Exposed ($n = 39/39$)		Total ($n = 72$)	
	Null[f] ($n = 18$)	Present[f] ($n = 15$)	Null ($n = 20$)	Present ($n = 19$)	Null ($n = 38$)	Present ($n = 34$)
(b) GSTM1 genotype						
Hyperdiploidy						
Chromosome 1	0.60 ± 0.10	0.96 ± 0.25	0.63 ± 0.16	0.84 ± 0.31	0.62 ± 0.09	0.89 ± 0.20
Chromosome 7	0.71 ± 0.15	0.90 ± 0.24	0.85 ± 0.20	1.04 ± 0.37	0.78 ± 0.13	0.98 ± 0.23
Chromosome 8	0.52 ± 0.09	0.91 ± 0.23	0.64 ± 0.15	1.03 ± 0.35	0.58 ± 0.09	0.98 ± 0.22
Chromosome 12	0.54 ± 0.09	1.04 ± 0.24	0.68 ± 0.19	0.85 ± 0.31	0.61 ± 0.11	0.93 ± 0.20
Structural changes						
Chromosome 8	0.20 ± 0.12	0.15 ± 0.08	0 ± 0	0.15 ± 0.07[g]	0.10 ± 0.06	0.16 ± 0.05
Chromosome 12	0.22 ± 0.13	0.28 ± 0.10	0.18 ± 0.12	0.18 ± 0.08	0.20 ± 0.09	0.22 ± 0.06
HPRT ($M_f \times 10^{-6}$)[d]	16.70 ± 2.74	22.18 ± 6.10	17.91 ± 1.79	27.02 ± 7.46	17.38 ± 1.55	24.92 ± 4.90
n_{HPRT}[e]	15	10	19	13	34	23

[a] *GSTT1* present as wild-type and heterozygote, and *GSTT1* null as homozygote mutant genotype.
[b] $P = 0.07$ by Wilcoxon Rank Sum W test.
[c] $P = 0.05$ by Wilcoxon Rank Sum W test.
[d] *HPRT* mutant frequency per 10^6 cells.
[e] Number of subjects that could be analyzed for *HPRT* in the exposure/genetic subgroup.
[f] *GSTM1* present as wild-type and heterozygote, and *GSTM1* null as homozygote mutant genotype.
[g] $P = 0.02$ by Wilcoxon Rank Sum W test.

Table 4
Effect of *GSTM1* and *GSTT1* genotypes on chromosomal damage and *HPRT* mutation

GST	GSTs genotype[a]			
	Null, present (25)[b]	Null, null (13)[b]	Present, present (19)[b]	Present, null (14)[b]
Hyperdiploidy				
Chromosome 1	0.59 ± 0.12[c]	0.67 ± 0.15	0.73 ± 0.1	1.17 ± 0.45
Chromosome 7	0.72 ± 0.17	0.91 ± 0.17	0.91 ± 0.20	1.15 ± 0.50
Chromosome 8	0.58 ± 0.12	0.59 ± 0.12	0.82 ± 0.16	1.27 ± 0.47
Chromosome 12	0.61 ± 0.15	0.62 ± 0.14	0.87 ± 0.16	1.09 ± 0.45
Structural changes				
Chromosome 8	0.06 ± 0.05	0.17 ± 0.15	0.24 ± 0.08	Constant
Chromosome 12	0.14 ± 0.10	0.31 ± 0.17	0.17 ± 0.08	0.26 ± 0.09
HPRT ($M_f \times 10^{-6}$)[d]	17.0 ± 1.89	18.1 ± 2.77	22.7 ± 4.41	30.8 ± 12.0
n_{HPRT}[e]	22	12	14	8

[a] *GSTs* present as wild-type and heterozygote, and *GSTs* null as homozygote mutant genotype.
[b] *GSTM1*, *GSTT1* (*n*).
[c] Mean frequency (%) ± standard error of mean.
[d] *HPRT* mutant frequency per 10^6 cells.
[e] Number of subjects that could be analyzed for *HPRT* in the exposure/genetic subgroup.

GSTT1. Twenty-seven (38%) of the 71 subjects genotyped were *GSTT1* null, similar to rates observed in other Chinese populations [23]. *GSTT1* genotype had no significant effect on the frequency of hyperdiploidy of chromosomes 1, 7, 8, or 12 or on the frequency of structural changes of chromosomes 8 and 12, both in controls and exposed (Table 3a).

Thirty-three of 38 controls and all 39 exposed were typed for *GSTM1*. Among controls, 18 subjects were null type and 15 were wild-type, while among exposed 20 were null type and 19 were wild-type, meaning that 38 out of 72 subjects (53%) were GSTM1 null, a frequency similar to that observed in other studies of Chinese populations [23]. *GSTM1* genotype generally had no effect on levels of hyperdiploidy or on structural changes in both controls and exposed (Table 3b).

Subjects who were *GSTT1* null and *GSTM1* present had the highest hyperdiploidy frequencies of all four chromosomes targeted and highest mutant frequencies for *HPRT* independent of BD exposure, whereas the opposite combination (*GSTT1* positive and *GSTM1* null) had the lowest frequencies of both genetic changes (Table 4). Thus, *GST* polymorphisms influenced the background frequency of genetic damage.

3.5. Effect of EPHX1 genotypes and corresponding phenotypes

The polymorphisms of *EPHX1*, particularly, for *EPHX1* Y113H were determined by both RFLP and gene sequencing methods. Following an initial analysis of *EPHX1* Y113H by RFLP, a paper describing a

Table 5
Genotypes and predicted activity of EPHX1 in the study population[a]

$EPHX1^{139}$	$EPHX1^{113}$		
	YY (33.7 wt.%) (Tyr/Tyr)	YH (45.5%) (Tyr/His)	HH (20.8%) (His/His)
HH (84.4 wt.%) (His/His)	Intermediate (20)[b]	Low (29)	Low (16)
HR (15.6%) (His/Arg)	High (6)	Intermediate (6)	Low (0)
RR[c] (0%) (Arg/Arg)	High (0)	High (0)	Intermediate (0)

[a] According to criteria developed by Benhamou et al. [26] and Viezzer et al. [27].
[b] Values in parentheses indicate number of subjects in the study with this activity level and combined genotype.
[c] No homozygous mutant RR subjects were detected in this study.

Table 6
Effect of *EPHX1* genotype and predicated phenotype on chromosome changes and *HPRT* mutation in BD-exposed workers ($n = 39$)

Genotype	*EPHX1*^{113} (Y → H)[a]		*EPHX1*^{139} (H → R)[b]		*EPHX1*$^{113/139}$	
	HH/YH ($n = 28$)	YY ($n = 11$)	HH ($n = 32$)	HR/RR ($n = 7$)	Low[c] ($n = 27$)	High[d] ($n = 12$)
Hyperdiploidy						
Chromosome 1	0.67 ± 0.22[e]	0.89 ± 0.25	0.62 ± 0.19	1.27 ± 0.26[f]	0.67 ± 0.22	0.88 ± 0.23
Chromosome 7	0.93 ± 0.27	0.99 ± 0.27	0.87 ± 0.24	1.30 ± 0.28[g]	0.93 ± 0.28	0.97 ± 0.25
Chromosome 8	0.81 ± 0.25	0.90 ± 0.22	0.74 ± 0.22	1.27 ± 0.26[f]	0.81 ± 0.26	0.89 ± 0.20
Chromosome 12	0.72 ± 0.23	0.88 ± 0.26	0.68 ± 0.21	1.17 ± 0.33	0.72 ± 0.24	0.87 ± 0.24
Structural changes						
Chromosome 8	0.07 ± 0.04	0.09 ± 0.07	0.08 ± 0.04	0.04 ± 0.04	0.07 ± 0.04	0.08 ± 0.06
Chromosome 12	0.17 ± 0.06	0.20 ± 0.20	0.14 ± 0.06	0.36 ± 0.31	0.18 ± 0.07	0.18 ± 0.18
HPRT ($M_f \times 10^{-6}$)[h]	22.4 ± 4.1	20.1 ± 3.4	22.8 ± 3.9	17.5 ± 2.9	22.5 ± 4.2	20.2 ± 2.9
n_{HPRT}[i]	25	6	26	5	24	7

[a] A transition of T to C in exon 3 of *EPHX1* changes residue 113 from tyrosine (Y) to histidine (H), which causes a decrease in the enzyme activity of 40%. YY denotes wild-type. YH and HH denote heterozygote and homozygote mutant genotypes, respectively.

[b] An A to G transition in exon 4 of *EPHX1* changes residue 139 from histidine (H) to arginine (R), which causes an increase the activity of *EPHX1* ~25%. HH therefore denotes the wild-type. HR and RR denote heterozygote and homozygote mutant genotypes, respectively, but no RR genotypes were detected.

[c] Low activity of *EPHX1* (includes slow and very slow) classified as indicated in [26,27].

[d] High activity of *EPHX1* includes high and intermediate activities (fast and normal) which are classified as indicated in [26,27].

[e] Mean frequency (%) ± standard error of mean.

[f] $P < 0.05$ by Wilcoxon Rank Sum W test.

[g] $P = 0.05$ by Wilcoxon Rank Sum W test.

[h] *HPRT* mutant frequency per 10^6 cells.

[i] Number of subjects that could be analyzed for *HPRT* in the exposure/genetic subgroup.

second nucleotide substitution close to the polymorphic site and within the sequence of the downstream primer, came to our attention [21]. The authors show that the primer mismatch causes reduced amplification efficiency, leading to some individuals heterozygous for His113/Tyr113 being incorrectly classified as homozygous for His113. We therefore re-analyzed the homozygous individuals identified in our study by sequencing. Indeed, of 33 individuals originally determined to be His113 by RFLP, 17 were shown to be heterozygous while 16 were confirmed as homozygous mutants. The corrected frequencies for the genotypes of *EPHX1* Y113H observed here (Table 5) are very similar to those described in other Asian populations, which were analyzed by methods not biased by the second polymorphism [24,25].

The *EPHX1* Y113H polymorphisms had no influence on chromosome damage or *HPRT* mutant frequency in either the exposed subjects (Table 6) or controls (data not shown). The *EPHX1* H139R polymorphism did, however, influence the level of hyperdiploidy in exposed subjects (Table 6), but not in controls (data not shown). In exposed subjects who had HR or RR alleles the level of hyperdiploidy of chromosomes 1, 7 and 8 were significantly higher than subjects with the HH genotype. This is somewhat counterintuitive as the HH genotype is associated with low to intermediate EPHX1 activity (Table 5) and therefore should theoretically afford less protection against BD epoxides. No effect of the H139R polymorphism on *HPRT* mutant frequency was observed (Table 6). When the two polymorphisms were combined as previously described to give predicted activities of EPHX1 [26,27], 45 of the study subjects had low predicted activity and 32 had intermediate to high activity (Table 5). Among the BD-exposed workers 27 had low and 12 had intermediate to high predicted EPHX1 activity. Intermediate to high EPHX1 activity did not lower any of the measured genotoxicity endpoints (Table 6), showing that in our study predicted EPHX1 activity did not influence genetic damage at low occupational BD exposures.

4. Discussion

We have measured chromosomal changes by FISH and *HPRT* gene mutant frequency in the peripheral blood lymphocytes of 39 BD polymer production workers and 38 unexposed controls in Yanshan, China. Various methods, including air monitoring, urinary metabolite assays and measurements of hemoglobin adducts were used to establish that the exposed workers were exposed to a median level of 2 ppm BD on a daily basis. We initially performed a transitional epidemiology study of 10 exposed and 10 controls using FISH to measure aneuploidy in interphase cells. Finding no difference (data not shown), we decided to employ a more sensitive FISH assay of chromosome damage in metaphase spreads and studied all workers and controls with available metaphase spreads in a double-blind fashion. BD-exposed workers did not have higher levels of aneuploidy or structural chromosome aberrations in the four chromosomes 1, 7, 8 and 12, examined using FISH. In fact, the levels of several numerical and structural changes among controls were slightly higher than those in exposed. We also found no increase in *HPRT* gene mutant frequency among the BD-exposed workers. These negative findings agree with recent reports from studies in the Czech Republic [15], which used classical methods and FISH to examine chromosomal changes, but not with some of the earlier literature [11,14].

An early report on a small number of workers from the Czech Republic and Portugal described no effect of BD on the level of chromosome aberrations, sister chromatid exchanges (SCEs) or micronuclei in peripheral blood on original analysis [12]. However, re-analysis with respect to *GST* genotypes showed that the *GSTT1* null BD-exposed workers had significantly higher aberration frequencies than the matched controls [12]. Further study of the same Czech Republic workers reported in 1998 showed significant elevations in chromosome aberrations and SCEs in the BD-exposed group (exposed on average $0.53\,mg/m^3 = 1.17\,ppm$) compared to controls, but no association with the *GSTT1* null genotype [14]. A recent expanded molecular epidemiological study of Czech Republic BD workers has measured the M1 and M2 urinary metabolites, as well as the HBVal and THBVal hemoglobin adducts as biomarkers of BD exposure, and determined if genotoxic effects on the gene or chromosome level occurred at the documented exposure levels [15]. The study included 24 BD monomer production workers (mean BD exposure: $0.642\,mg/m^3 = 1.42\,ppm$), 34 polymerization workers (mean BD exposure: $1.794\,mg/m^3 = 3.97\,ppm$) and 25 controls (mean BD exposure: $0.023\,mg/m^3 = 0.05\,ppm$). Urinary M1 and M2 metabolite and HB-Val and THBVal hemoglobin adduct concentrations were all significantly correlated with BD exposure levels, with adducts being the most highly associated. Background (control) THBVal adduct concentrations were higher than previously observed in other control populations (North American and Chinese), suggesting some endogenous BD source for the metabolite. There was no evidence, however, of increased chromosome damage as assessed by FISH and classical methods at this level of BD exposure. Metabolic genotypes also did not influence any of these findings on genotoxicity. These more recent findings agree with those found in the present study. They do not preclude BD from producing chromosome damage at higher levels than we have studied, but they do show that chromosome damage is not detectable in the 0.5 to 2 ppm range of exposure.

Our negative findings on *HPRT* mutations and BD exposure are in contrast to positive findings from one laboratory on BD-exposed workers at a Texas facility using the autoradiography assay to measure mutations in *HPRT* [10]. BD exposures of the workers in this study were 3.5 ± 7.5 ppm in the high-exposure areas. Another study by this same group, using the *HPRT* cloning assay, was positive according to the authors and the mutational spectrum data were compatible with the *HPRT* mutations being induced by BD, in which an excess of deletions were found [28]. By contrast, our studies in BD-exposed Chinese workers [7] and those in Czech workers by other groups [11,15], using the cloning assay, have failed to find increases in *HPRT* mutations even though the level of BD exposure was similar to the Texas studies. Furthermore, no increases in chromosome aberrations were found in the Texas facilities, although a "Challenge Assay," in which cells were exposed to gamma rays in G1 in vitro, was positive [29]. The latter could perhaps reflect the induction of abnormal DNA repair after exposure to BD [13].

In these same Texas facilities, Abdel-Rahman et al. reported that the Y113H polymorphism in *EPHX1*

gene was correlated with increased *HPRT* variant frequency following BD exposure in a styrene/butadiene rubber facility [16]. An increased frequency of *HPRT* mutation was observed in the lymphocytes of workers who were exposed to higher levels of BD (typically >150 ppb, with some approaching 10 ppm) and also had at least one His allele (H/H or Y/H genotypes). In the present study, we examined this Y113H polymorphism in *EPHX1* as well as another functional polymorphic variant at position 139 (H139R), so as to provide a more complete phenotypic picture of this important enzyme in BD metabolism. Abdel-Rahman et al. have also recently performed a similar analysis in their study and have presented findings that support their earlier conclusion that *EPHX1* polymorphisms influence BD genotoxicity in humans [30]. They reported that *EPHX1* polymorphisms that are predicted to confer low EPHX1 activity elevated the *HPRT* variant frequency in workers exposed to >150 ppb BD by two- to three-fold in comparison to that found in workers with intermediate to high EPHX1 activity. In contrast, we found no effect of either genetic polymorphisms in *EPHX1* or predicted EPHX1 activity on *HPRT* gene mutant frequency following BD exposure. The reasons for this difference are unclear, but the recent findings of Abdel-Rahman et al. [30] are based on a comparison of only seven BD-exposed workers with low EPHX1 activities against nine with intermediate to high activity. Our negative findings are based upon a larger population comparing 27 BD-exposed workers with low activity against 12 with intermediate to high EPHX1 activity. Only additional larger studies can resolve whether or not EPHX1 plays any role in susceptibility to BD genotoxicity in humans, but our results do not corroborate those of Abdel-Rahman et al.

Our study also showed no influence of either *GSTT1* or *GSTM1* genotype on BD-induced aneuploidy and *HPRT* gene mutation. We, therefore, combined the exposed and control groups to examine the overall influence of *GSTT1* and *GSTM1* on the background level of chromosome and gene mutations (Table 4). This combining of the data does have some drawbacks, in that the subgroups become small and the case-control matching may be lost. However, we expected that *GSTT1* null and *GSTM1* null ($GSTT1^-/GSTM1^-$) individuals would have the highest mutation rates and those positive for both *GST*s the lowest levels of damage. To our surprise individuals who were *GSTT1* null and *GSTM1* positive ($GSTT1^-/GSTM1^+$) had the highest levels of genetic damage, whereas $GSTT1^+/GSTM1^-$ individuals had the lowest. This suggests that $GSTT1^-/GSTM1^+$ individuals may be at higher risk of cancer, an observation that requires further investigation. Studies have shown that *GSTT1* null individuals are at higher risk of certain cancers but the influence of *GSTM1* is less clear. Previous studies on baseline chromosome aberrations have also shown slightly higher frequencies in $GSTT1^-$ individuals [31], but again the influence of *GSTM1* is less obvious although a recent study has shown higher levels of aberrations in $GSTM1^+$ individuals [32].

In summary, our data show no increase in chromosomal aberrations or *HPRT* mutations among workers exposed to BD, even in potentially susceptible genetic subgroups. This is contrast to the findings of other studies in Texas of similar size and BD exposure level. However, our findings in China do support those from studies conducted in the Czech Republic. Together, the studies of Chinese and Czech workers suggest that low levels of occupational BD exposure do not pose a significant risk of genetic damage.

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